



## The Effects of the Herbicide Asulam on the Gametophytes of *Pteridium aquilinum*, *Cryptogramma crispa* and *Dryopteris filix-mas*

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Fern spores were germinated, grown and exposed to different concentrations of asulam (the active ingredient of Asulox) in liquid culture. Gametophytes of *Pteridium aquilinum* [L. (Kuhn)] (bracken) were exposed to 100 g l<sup>-1</sup> for 24 h at the following three stages: germination; during filamentous growth; and after transition had occurred. This treatment severely inhibited germination and killed photosynthetic gametophytes outright. Gametophytes of all species tested [*Pteridium aquilinum*, *Cryptogramma crispa* (L.) Hook and *Dryopteris filix-mas* (L.) Schott] varied in their responses to lower concentrations. This variation was in terms of the response of the gametophytes and the numbers of gametophytes showing each response. Some gametophytes suffered 100% cell mortality, but in others some cells died and some survived, while the remainder of the gametophytes suffered no cell mortality. The cells affected varied from gametophyte to gametophyte at the same dose and the proportions of gametophytes showing each response changed with concentration, mortality increasing with increasing concentration. *Pteridium* showed lower levels of mortality at each concentration than either of the other species. The bioassay system described provides a rapid laboratory-based method to screen for Asulox susceptibility of fern gametophytes in comparison with *Pteridium*.

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**Key words:** *Pteridium aquilinum* (bracken), *Cryptogramma crispa* (parsley fern), *Dryopteris filix-mas* (male fern), gametophyte, Asulox, asulam, herbicide.

### INTRODUCTION

Given the problems caused by *Pteridium* that have been outlined in the editorial and other papers in this issue, it is unsurprising that bracken control has been well studied (see Fletcher and Kirkwood, 1979 for review). Of the methods available, chemical spraying is the most widely used today, and of the chemicals available, Asulox is the only herbicide approved for aerial spraying in the United Kingdom (Anon, a). Asulox is a 40% w/v solution of the sodium salt of asulam, which is the active ingredient of the chemical (Heywood, 1982).

The general mode of action of asulam in bracken sporophytes is well understood. It is taken up into the plant and transported systemically to sink areas (Veerasekaran, Kirkwood and Fletcher, 1977a; Fletcher and Kirkwood, 1979). If applied late in the growing season, these sink areas are the rhizomes. Once in the rhizomes, asulam acts in typical carbamate fashion to inhibit mRNA and protein synthesis and therefore cell growth and division (Veerasekaran, Kirkwood and Fletcher, 1976, 1977b).

Although asulam (in the form of Asulox) is used in large amounts in the control of bracken, it was initially marketed as a control for *Rumex acetosa* (L.), an angiosperm, demonstrating that it is not specific to bracken. Despite this, relatively little work has been carried out on the effects

of asulam on other British species, particularly other ferns. Horrill, Thomson and Dale (1978) investigated the response of a number of plant species by directly spraying the plants (which had been removed from the wild and cultivated) with different concentrations of asulam, but only tested two ferns, viz *Dryopteris dilatata* (Hoffm.) A. Gray and *Blechnum spicant* (L.) Roth. A study by Marrs and Frost (1996) looked at one other fern, the tropical species *Adiantum pubescens* (Schkuhr). The study by Horrill *et al.* in 1978 showed great variation in the response of treated fern sporophytes, due to growth stage differences between plants and the size of the plants, especially in rhizomatous species such as bracken.

There is therefore a need for a rapid and repeatable assay to determine the susceptibility of non-target plants, particularly fern species, which are expected to be particularly at risk (Robinson and Page, 2000). Gametophytes offer one way to do this, as they are relatively easy to culture in the laboratory (e.g. Dyer, 1983; Douglas and Sheffield, 1992; Gray, 1998) and are genetically identical to sporophytes. A liquid culture system offers exact control over exposure concentrations and times. The doses used in this study were initially chosen to reflect the concentrations used in the spray tanks of a helicopter, i.e. 100 g l<sup>-1</sup>. Spores or gametophytes could be struck and engulfed by a droplet at this concentration. However, concentrations are difficult to compare with the dose of asulam recommended for spraying sporophyte stands in the UK (4.4 kg asulam ha<sup>-1</sup>).

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No previous study has addressed the problem of gametophyte susceptibility or considered its possible relation to sporophyte sensitivity either in terms of producing an assay or in conservation terms. This latter aspect is an important issue in terms of the rarer ferns, as any loss of diversity suffered as a result of the impact of herbicide on plants growing in or near to bracken stands must be avoided if possible. This study therefore aimed to assess gametophyte susceptibility of bracken and other fern species and to provide a baseline for a comparison of the effects of asulam on ferns.

## MATERIALS AND METHODS

Each experiment was carried out separately and with its own internal control (i.e. 0 g l<sup>-1</sup> asulam for each species present in the experiment). All doses in each experiment were replicated four times and a mean value calculated for the variables counted. (In expt 3, the only one in which control mortality was significantly higher than zero, values for asulam doses were corrected by subtracting the mean mortality levels in the controls from the mortality values in the exposed flask. This removed the effects of asulam-independent mortality from the data. This applies solely to the data presented in Fig. 6.)

### Source of spores

All bracken spores were from a sample collected in August 1997 from a natural population of subspecies *aquilinum*, variety *aquilinum* near Millgate Farm, South Manchester, UK. The spores of *Dryopteris filix-mas* were collected from plants growing at the Botanical Experimental Grounds, University of Manchester, UK in August 1985 and the *Cryptogramma crispa* spores were collected in August 1990 from a natural population growing near Wanlothead, Dumfries and Galloway, UK.

### Spore preparation

Spores were weighed to give a final spore density of 2000 spores per ml in the experimental flasks. They were then suspended in the appropriate volume of 1:1 sterile distilled H<sub>2</sub>O and sterile 0.5% high viscosity carboxymethylcellulose (CMC) solution and allowed to start germinating at room temperature in the dark for 24 h. The spore suspension was then made up, with the same solution, to a final volume sufficient to provide 2 ml of this suspension for each conical flask used in the experiment. This amount was pipetted into each sterilized 250 ml flask and the total volume per flask made up to 100 ml by the addition of autoclaved Moore's medium (Moore, 1903). The flasks were placed on orbital shakers rotating at 100 rpm in a walk-in growth room at 20 ± 2°C, photon flux density 150 µmol m<sup>-2</sup> s<sup>-1</sup> on a 12 h light/dark cycle.

### Exposure Experiment 1 (Pteridium only)

Gametophytes were exposed to asulam at one of three stages: 'germination'; during filamentous growth; and after

transition to two-dimensional growth. These stages corresponded to 2 d, 7 d and 14 d after imbibition. Prior to exposure, the media volume was checked and if necessary made up to 100 ml with Moore's medium to compensate for losses due to evaporation on shakers. To expose the spores, the appropriate amount of Asulox (at 400 g l<sup>-1</sup> asulam) was added to each flask to give a final concentration of 100 g l<sup>-1</sup> asulam, and the flasks were returned to the shaker for 24 h.

### Experiment 2 (Pteridium only)

Filamentous gametophytes were exposed as above to concentrations from 0–75 g l<sup>-1</sup> asulam at 12.5 g l<sup>-1</sup> intervals and from 25–37.5 g l<sup>-1</sup> at 2.5 g l<sup>-1</sup> intervals, again for 24 h (sterile dH<sub>2</sub>O was added to the lower concentration flasks to equalize the volumes in each flask).

### Experiment 3

Filamentous gametophytes (7-d-old *Pteridium*, 10-d-old *Dryopteris* and 12-d-old *Cryptogramma*) were exposed as above. To compare the response of the three species, all three were exposed to 0, 1, 10 or 25 g l<sup>-1</sup> asulam for 24 h.

### Resuspension of gametophytes

The spores or gametophytes were removed from the asulam/Moore's solution using a Sartorius filter. A 47 mm diameter, 0.2 µm pore diameter membrane filter was placed in the filter holder, the Sartorius filter assembled and connected to a vacuum pump and the solution sucked out, leaving the material on the membrane filter. The material was then rinsed and filtered with three 50 ml aliquots of sterile dH<sub>2</sub>O. The membrane filter was removed and the material rinsed off into a sterile beaker using fresh Moore's medium. The suspension was made up to a final volume of 100 ml with Moore's medium and transferred to a flask. The flasks were returned to the shaker for 7 d.

### Sampling

From each replicate in expt 1, a sample of 1000 spores was counted to assess germination. In subsequent experiments, 250 germinated gametophytes of bracken and 300 of other species were sampled to assess the effects on photosynthetic gametophytes. Counting was carried out in Petri dishes to assess the germination stage and in a Sedgewick Rafter Chamber for the gametophyte stage. Spores were considered to have germinated if they had produced a rhizoid, otherwise they were counted as ungerminated. The gametophytes exposed to 100 g l<sup>-1</sup> asulam were assessed on their survival, i.e. if they had any living cells remaining. Each of the gametophytes exposed to lower concentrations was placed into one of the following classes: all cells dead (total mortality); some cells dead (partial mortality); all cells alive (total survival); and the numbers in each class recorded.

### Analysis

The mean percentage per treatment (total percentage in each class divided by the number of replicate samples) of each class of spore or gametophyte was calculated and plotted for each concentration. Statistical comparison used *t*-tests on the asine transformed percentage values from each flask. Significance was assessed at the 95% confidence level.

## RESULTS

Asulam at  $100 \text{ g l}^{-1}$  for 24 h (expt 1) inhibited *Pteridium* spore germination significantly, although inhibition was not complete (Fig. 1). At the same concentration, all photosynthetic gametophytes sampled suffered total cell mortality (Fig. 2).

At lower concentrations (expts 2 and 3), the effects of asulam on *Pteridium* varied both between concentrations and between gametophytes at the same concentration (Fig. 3). Some gametophytes suffered total cell mortality, others only partial, the remainder were unaffected. The cells killed by asulam were not the same in all gametophytes; some were at the base of the gametophyte, while others were in the middle or at the tip. There was no pattern to the cells killed in relation to either concentration or growth stage. However, there was a relationship between the

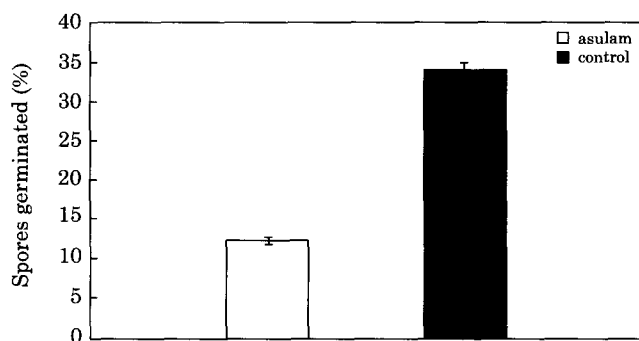


FIG. 1. Bar chart showing germination in exposed and control samples of *Pteridium* spores exposed to  $100 \text{ g l}^{-1}$  asulam for 24 h at 2 d from the start of imbibition. The difference in germination levels was significant above the 95% confidence limit.

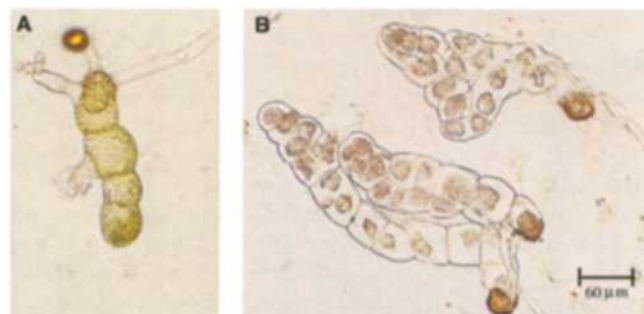


FIG. 2. *Pteridium*. A, Typical gametophyte from the control treatment, healthy and photosynthetic. B, Gametophytes exposed to  $100 \text{ g l}^{-1}$  asulam at 7-d-old. 100% of the gametophytes had suffered total cell mortality.

proportion of each gametophyte type and concentration. With increasing concentration, a response was seen between  $25 \text{ g l}^{-1}$  and  $37.5 \text{ g l}^{-1}$  (Fig. 4) with the proportion of gametophytes with total and partial cell mortality increasing dramatically at this point, and a corresponding fall in the numbers of gametophytes with total cell survival. When concentrations between these two points were studied (Fig. 5), a large change was seen between  $27.5 \text{ g l}^{-1}$  and  $30 \text{ g l}^{-1}$  in terms of total survival and total mortality, but not in terms of partial mortality.

The other two species tested, *Dryopteris filix-mas* and *Cryptogramma crista*, were significantly more sensitive to asulam at concentrations of  $25 \text{ g l}^{-1}$  and  $10 \text{ g l}^{-1}$ , respectively in terms of total cell mortality. *Cryptogramma crista* was also significantly more sensitive at  $1 \text{ g l}^{-1}$  than *Pteridium* (Fig. 6).



FIG. 3. The variable response of one sample of *Pteridium* gametophytes exposed at 7-d-old to the same concentration of asulam. Micrograph taken 7 d after exposure. Gametophyte A suffered total cell mortality, while B and C had some dead cells and some surviving cells.

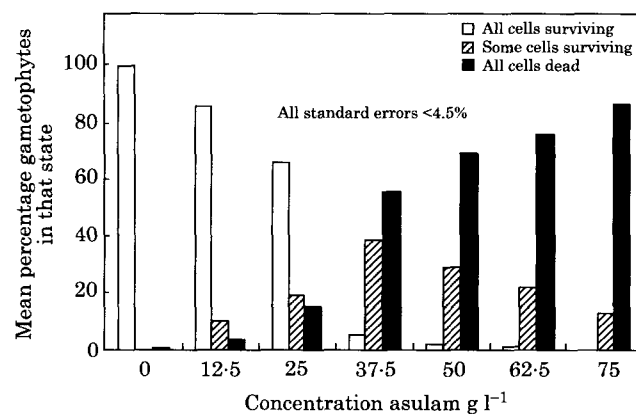


FIG. 4. Bar chart showing the percentages of *Pteridium* gametophytes in each state for concentrations between  $0 \text{ g l}^{-1}$  and  $75 \text{ g l}^{-1}$ . Note the large change in gametophyte response between  $25 \text{ g l}^{-1}$  and  $37 \text{ g l}^{-1}$  (a difference significant at 95% confidence level).

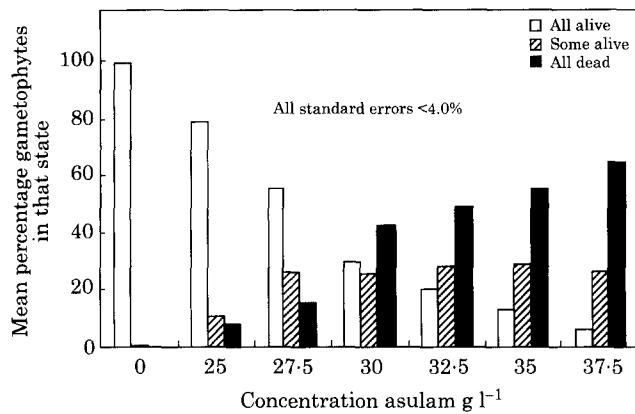


FIG. 5. Bar chart showing the percentages of gametophytes in each state for asulam concentrations between 25 g l<sup>-1</sup> and 37 g l<sup>-1</sup>. Concentrations of note were 30 g l<sup>-1</sup>, as all cells dead becomes the dominant gametophyte type and 32.5 g l<sup>-1</sup>, at which there was 50% mortality.

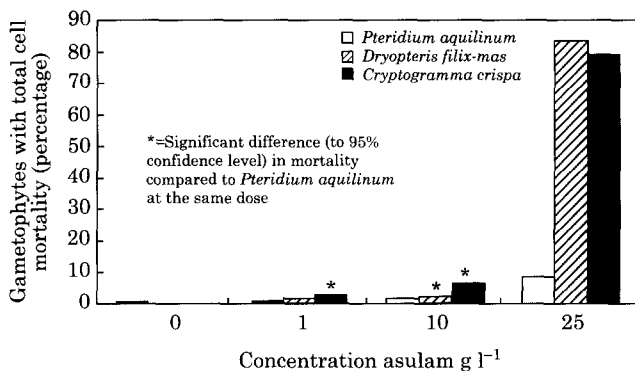


FIG. 6. Bar chart comparing the susceptibility of filamentous gametophytes of *Dryopteris filix-mas*, *Cryptogramma crispa* and *Pteridium aquilinum* at three asulam concentrations. N.B. The *Pteridium aquilinum* data was adjusted to remove asulam independent mortality (i.e. mortality in the control flasks).

## DISCUSSION

When considering the germination data it is necessary to remember that both before and after being stored, fern spores often have widely different percentages of germination and can take different amounts of time to start germinating (Conway, 1949). It is therefore likely that the spores in the flasks that germinated after exposure to herbicide were the slower-germinating spores which survived because the asulam failed to penetrate the spore wall, which was intact throughout the exposure period. It seems likely that asulam was totally effective against all the germinating spores. The corollary is that spores of bracken, and perhaps of other species, which are not imbibed at the time of exposure should not be damaged by exposure to asulam. The effects of 100 g l<sup>-1</sup> asulam on both spores and gametophytes suggest any such material hit by spray droplets in the field will be severely affected.

At lower concentrations, however, bracken gametophytes exhibit differential responses—some survive, others do not. Amongst the survivors, some have all their cells intact while

others show some cell mortality. Given that asulam inhibits cell division in sporophytes, and the fact it is transported to sink areas (Veerasekaran *et al.*, 1977a), it would be reasonable to expect that the gametophytic cells mainly affected would be the dividing cells. These are the tip cells of filamentous gametophytes (the main site of cell division at this stage) and the cells in the centre towards the front edge of the gametophytes after transition to a prothallus. However, the gametophytic cells appeared to be affected at random, with the dividing cells no more frequently affected than others. While this variable response was unexpected in terms of the mode of action of asulam, it might be another expression of the variable responses reported for bracken sporophytes following spraying with Asulox (e.g. Le Duc *et al.*, this volume). If the type and location of cells killed by asulam is also not constant in the sporophyte, then the effects of exposure will vary depending on which cells of the sporophyte are killed. If the cells killed are not involved in organ generation the cost to the sporophyte will be slight. If the cells killed are important to, for example, frond regeneration, then the effects may be more severe. This type of response was reported by Horrill *et al.* (1978) in relation to *Dryopteris dilatata*.

From a conservation point of view, our results suggest that chance might play a large part in determining the effects of asulam on exposed plants and therefore any exposure could be a threat. This problem will become more important if other species are not only more susceptible than bracken but exhibit the same sharp changes in response to changes in concentration seen in *Pteridium*.

In terms of accidental exposures of plants in the field, the highest concentrations tested here are of no concern, as any rare ferns should not be directly sprayed if the statutory requirements governing spraying events are observed. These requirements include consultation with the Environment Agency and any appropriate nature conservancy agency (Anon, *b*). However, spray drift (droplets of the sprayed chemical carried by the wind out of the sprayed area) is an issue, especially if other fern species show lower tolerances to asulam than bracken. The comparison shown in Fig. 6 confirms that, if the bioassay results are representative of the response of the species in field conditions, then there are indeed some species that are more susceptible to asulam than bracken. The spores used in this study were not all at the same age, but if similar responses are shown by spores of the same age tested at the same time, this would suggest that the utmost care must be taken around rare fern species when spraying with Asulox. While bioassays in the field using damage to *Rumex* at known distances from the spray zone (Robinson, 1998) have shown that the existing buffer zones for spraying events are more than adequate, these data relate to *Rumex*. The relationship of these data to other species, particularly ferns, and indeed of the data presented in this paper to the sporophytic stages of ferns, is not known. Until this is no longer the case, any conclusions must be treated carefully and any decisions must always err on the side of caution.

This assay offers a relatively fast and easy way to assess the relation of other ferns to bracken in terms of their susceptibility to asulam. The results gained so far do

suggest that direct comparisons between species are possible and that results gained by using this system may be consistent enough to calculate a type of  $LC_{50}$  value for each species. An  $LC_{50}$  is the concentration at which 50% of individuals in the exposed sample are killed. As asulam acts at a cellular level, its effectiveness is likely to be linked to the concentration within the cells, not to the amount of active ingredient sprayed per unit area. Therefore, looking at a system such as the one outlined in this paper may offer some insight into the effects of this popular herbicide on fern sporophytes, although spray composition and amount of capture must be considered in field conditions.

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